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Characterization of a cross-reactive linear epitope in human genogroup I and bovine genogroup III norovirus capsid proteins

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Abstract

The Southampton norovirus (SV) capsid protein was expressed as VLPs by recombinant baculoviruses in insect cells and was used to immunize mice for the production of monoclonal antibodies (mAbs). One mAb, CM54, showed broad cross-reactivity to genogroup I (GI) noroviruses, but was not reactive to GII capsid proteins. Interestingly mAb CM54 reacted to a bovine norovirus capsid protein. Immunoblot analysis indicated the binding site for CM54 was located in the shell domain between amino acid residues 102–225 of the SV capsid protein. The epitope was mapped to high resolution using a peptide array and was located to the sequence LEDVRN at amino acid residues 162–167. Alignment of norovirus capsid protein sequences confirmed the epitope sequence was common to particular groups of human and bovine noroviruses. Modeling of the epitope onto the recombinant NV capsid protein revealed it was located to the inner surface of the shell domain.

Keywords: Norovirus; Capsid; Monoclonal antibody; Epitope

Introduction

Caliciviruses cause a variety of diseases in humans and animals. On the basis of phylogenetic variation and genome organization, members of the Caliciviridae have been divided into four distinct genera; vesiviruses, lagoviruses, noroviruses and sapoviruses (Green et al., 2000). Recently, on the basis of phylogenetic analyses, a fifth calicivirus genus has been proposed for the bovine viruses Nebraska (NB) (Smiley et al., 2002) and Newbury agent-1 (NA-1) (Oliver et al., 2006a). Viruses in the genera Norovirus and Sapovirus are the causative agents of non-bacterial gastroenteritis in man, cattle and swine (Liu et al., 1999; Sugieda et al., 1998; Kapikian, 1994; Bridger et al., 1984). The noroviruses have been subdivided into five genogroups (Fankhauser et al., 2002): viruses in genogroups I, II and IV are associated with humans; genogroup III noroviruses are associated with bovines and genogroup V is represented by the newly discovered mouse norovirus (MNV-1) (Karst et al., 2003).

Norovirus research has been greatly hindered by the inability to propagate noroviruses in cell culture and the low number of viruses in clinical specimens. To date, studies on human noroviruses have relied on clinical specimens and materials obtained from infected volunteers. Noroviruses are highly infectious and spread rapidly by person to person in closed and semiclosed environments (Green et al., 2002; Marx et al., 1999) or through the contamination of food and water (Khan et al.,

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1994; Cramer et al., 2003; Pontefract et al., 1993; Fleissner et al., 1989; Perz et al., 2000). Traditionally diagnosis of norovirus infection has relied on the observation of virus particles by EM (Caul and Appleton, 1982). However, a significant advance in studying the noroviruses came with the discovery that expression of the prototype Norwalk virus (NV) capsid protein in insect cells, where it forms virus-like particles (VLPs) (Jiang et al., 1992). Subsequently, capsid proteins from a number of different noroviruses have been expressed in insect cells (Jiang et al., 1995; Kobayashi et al., 2000b; Dingle et al., 1995; Deng et al., 2003; Green et al., 1997) and all assemble to form VLPs that are morphologically and antigenically indistinguishable from native viruses (Green et al., 1993). The X-ray crystallographic structure of the NV capsid protein has been resolved to 3.2 Å and showed that the capsid consists of 90 dimers of a single protein with a $T=3$ icosahedral symmetry. A single capsid protein has two principal domains, the shell domain (amino acid residues 1–225) and the protruding domain (P), which is divided into two subdomains, P1 (amino acid residues 226–278 and 406–530) and P2 (amino acid residues 279–405), with the P2 subdomain most protruding. It has been suggested that the P2 subdomain is highly antigenic and probably responsible for determinants of strain specificity (Prasad et al., 1999). Norovirus VLPs are now an abundant and renewable source of antigen and have been used to generate specific antisera for use in antigen detection enzyme-linked immunosorbent assays (Vipond et al., 2000; Graham et al., 1994; Karst et al., 2003; Lewis et al., 1997; Honma et al., 1998; Deng et al., 2003; Kobayashi et al., 2000a).

Monoclonal antibodies (mAbs) have been raised against recombinant norovirus capsid proteins (Treanor et al., 1988; Yoda et al., 2000, 2001; Herrmann et al., 1995; Hardy et al., 1996). mAbs with different binding characteristics have been reported and an epitope from one mAb (that binds the recombinant NV capsid protein) has been located between amino acid residues 457–530 in the P1 domain, common to genogroup I noroviruses (Hardy et al., 1996; Hale et al., 2000). In a similar study, Yoda et al. characterized an epitope in the shell domain recognized by a genogroup cross-reactive mAb. Recently, a cross-reactive epitope present in GIII and GII/3 noroviruses was reported (Oliver et al., 2006b).

The purpose of this work was to identify mAbs showing a broad reactivity to noroviruses and to locate cross reactive epitopes on the capsid protein.

Results

Expression of capsid proteins in insect cells

The capsid protein of a novel GI/5 norovirus (Babbacombe isolate (Bab)), which shares 74.5% amino acid sequence identity with the SV capsid protein, was cloned and expressed as VLPs. The r-Bab capsid protein was expressed in insect cells and a time course of expression indicated that r-Bab capsid protein was detectable in infected cells from day 2 onwards. The r-Bab capsid protein was present as a doublet at 52 and 59 kDa and these proteins were exported to the cell culture supernatant from day 4 onwards (data not shown). r-Bab VLPs were purified by density gradient centrifugation and gradient fractions with a buoyant density of 1.34 g/ml contained VLPs as visualized by EM.

Expression of capsid proteins in E. coli

To increase the probability of isolating a monoclonal antibody reactive to a linear epitope, GST capsid fusion proteins were generated from the capsid genes for GI, GII and GIII noroviruses as described by Oliver et al. (2006b). Similarly a series of overlapping SV capsid protein fragments were expressed fused to GST in *E. coli*.

Antibodies to recombinant norovirus capsid proteins

Preliminary experiments screening hybridoma supernatants indicated that a large proportion of the hybridoma clones were producing antibodies to highly immunogenic baculovirus and insect cell antigens. Large numbers of selected hybridomas were found to secrete antibodies reactive with a 60-kDa baculovirus protein which was presumed to be the viral chitinase. This protein has a similar molecular weight to the norovirus capsid protein (data not shown) confusing interpretation of antibody reactivity by immunoblot analysis. These findings indicated that purified VLP preparations were contaminated with highly immunogenic baculovirus components. To circumvent this problem, we sought to produce an alternative source of norovirus capsid protein devoid of insect cell and baculovirus antigens for screening hybridomas secreting cross-reacting mAbs. For this purpose, the SV capsid protein fused to GST was expressed in *E. coli* using the vector pGex4T1. Analysis of bacterial cell cultures post-induction indicated that the SV-GST fusion protein was expressed as insoluble inclusion bodies. Purification of the SV-GST fusion protein was achieved using "Bugbuster" (Novagen, UK) and the yield of protein was estimated to be 750 μg/100 ml culture.

Mice were immunized with r-SV VLPs and hybridomas were screened by direct ELISA to SV-GST fusion protein. The selection of hybridomas based on their reactivity to SV-GST expressed and purified from *E. coli* was performed both to avoid the selection of mAbs reacting to baculovirus antigens and to increase the probability of selecting mAbs to linear epitopes. Hybridomas were cloned by terminal dilution and to ensure reactivity to authentic epitopes on the SV capsid protein, supernatants from the dilutions were tested in radioimmune precipitation assays to SV capsid protein expressed by in vitro transcription/translation. A single hybridoma (CM54) with the required binding characteristics was selected for further study.

A cross-reacting genogroup-specific monoclonal antibody

mAb CM54 was isotyped as IgM using the Mouse Typer® Sub-isotyping Kit (Bio-Rad, UK). To investigate the binding characteristics of mAb CM54, it was screened by direct ELISA for cross-reactivity to a panel of recombinant norovirus capsid proteins representing genogroups I–III (SV, Bab, DSV, NV,

Leeds, HwV, LV (Pelosi et al., 1999), TV, SMA, MD, JV (Deng et al., 2003) and NA-2 (Oliver et al., 2006b). mAb CM54 reacted to GI norovirus capsid proteins (SV (GI/1), Bab (GI/5), DSV (GI/3) and NV (GI/2)) and NA-2 capsid protein (GIII/2), but was non-reactive to GII norovirus capsid proteins or JV capsid protein (GIII/1). To confirm the pattern of cross-reactivity, mAb CM54 was used to immunoprecipitate the SV and Bab capsid proteins produced by in vitro transcription/translation. mAb CM54 immunoprecipitated the 58-kDa SV capsid protein and the 59-kDa Bab capsid protein (data not shown). The binding characteristics of mAb CM54 were investigated further by immunoblot (Fig. 1) against a panel of GI recombinant capsid proteins representing the major genetic clusters, the predominant Lordsdale GII/4 norovirus r-LV (Ando et al., 2000) and GIII/2 norovirus r-NA-2.

mAb CM54 reacted by immunoblot to the GI norovirus capsid proteins and GIII/2 norovirus NA-2 capsid protein, but did not bind to the LV (GII/4) capsid protein, indicating that mAb CM54 recognizes a continuous (linear) epitope common to GI and GIII/2 norovirus capsid proteins.

mAb CM54 was used to probe the antigenic structure of the SV capsid protein. A series of overlapping SV capsid protein fragments (SVF1, SVF2 and SVF3) were expressed fused to GST in *E. coli*. mAb CM54 reacted by immunoblot to SVF1 (amino acid residues 1–225) but not SVF2 or SVF3 (data not shown). The cDNA for SVF1 was cloned as two overlapping halves to produce plasmids expressing SV capsid protein fragments 4 and 5 (SVF4 and SVF5 respectively). mAb CM54 reacted by immunoblot to SVF5 (amino acid residues 102–225) (Fig. 2), but not SVF4 and showed no reactivity to expressed GST. The pattern of reactivity towards the SV capsid protein fragments showed that mAb CM54 recognized an epitope in the shell domain of the GI norovirus capsid.

High-resolution mapping of the epitope recognized by mAb CM54

To map at high-resolution the epitope recognized by mAb CM54, a peptide array was designed for amino acids 102–225 of the SV capsid protein. The peptide array consisted of dodecamers overlapped by three amino acids to resolve the epitope to a minimum of six amino acid residues. mAb CM54 bound to six peptides, 9 to 11 and 19 to 21 that corresponded to overlapping amino acid residues ^{132}FTSSSL^{137} and ^{162}LEDVRN^{167} in the SV capsid protein (Fig. 3A). The reactivity of CM54 to the predominant epitope LEDVRN was approximately 8-fold higher compared to the reactivity to FTSSSL. The two CM54 epitopes had a close proximity to one another in the capsid monomer as determined by molecular modeling, suggesting that the CM54 antibody recognized both linear and a conformational epitope. The FTSSSL and LEDVRN epitopes were located inside the norovirus capsid shown by macromolecular models of the capsid protein for the 5-fold axis of symmetry (Figs. 3B and C).

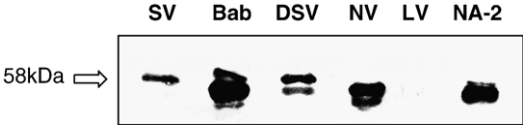

Fig. 1. Immunoblot of recombinant norovirus capsid proteins. Purified capsid proteins expressed in insect cells from recombinant baculoviruses were separated by SDS-PAGE and transferred to a nitrocellulose membrane which was reacted with the hybridoma cell culture supernatant of mAb CM54. Lanes 1–4 are from genogroup I noroviruses: Southampton virus (SV GI/1), Babbacombe virus (Bab GI/5), Desert Shield virus (DSV GI/3) and Norwalk virus (NV GI/1), lane 5 genogroup II Lordsdale virus (LV GII/4) and the final lane, lane 6 genogroup III Newbury Agent-2 (NA-2 GIII/2). The arrow shows the size of the recombinant SV capsid protein. mAb CM54 reacted with the GI norovirus capsid proteins and GIII/2 capsid protein, however, showed no reactivity to GII (LV) capsid protein.

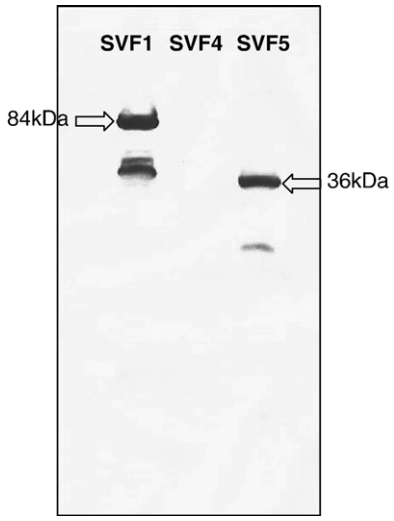

Fig. 2. Immunoblot of SV capsid proteins expressed in *E. coli*. Recombinant fragments of Southampton virus (SV) capsid protein fused to GST and expressed in *E. coli* were separated by SDS-PAGE and transferred to a nitrocellulose membrane which was reacted with hybridoma cell culture supernatant of mAb CM54. Tracks SVF1, SVF4 and SVF5 are cellular fractions of *E. coli* expressing fragments of the SV capsid protein post induction. mAb CM54 reacted with fragments SVF1 (amino acid residues 1–225) and SVF5 (amino acid residues 102–225), however, showed no reactivity to SVF4 (amino acid residues 1–124). The arrows indicate the estimated molecular weights for the two fusion proteins SVF1 and SVF5 respectively.

Norovirus capsid protein sequences available from Genbank were aligned to determine the distribution of this epitope within the Norovirus genus. The predominant epitope LEDVRN was conserved in GI, but not GII noroviruses (Fig. 4). Interestingly, the "epitope" sequence was not conserved in the JV (GIII/1) capsid protein, but conserved in the NA-2 (GIII/2) capsid protein, confirming the ELISA and immunoblot results. The FTSSSL component of the epitope showed little amino acid conservation for this region of the GII and GIII norovirus capsid proteins (data not shown).

Discussion

In this study, we used immunization with SV VLPs to produce a hybridoma secreting a mAb (CM54) that recognized an epitope common to GI and GIII/2 norovirus capsid proteins. mAb CM54 was selected based on its reactivity by direct ELISA with a complete SV capsid-GST fusion protein to help facilitate the

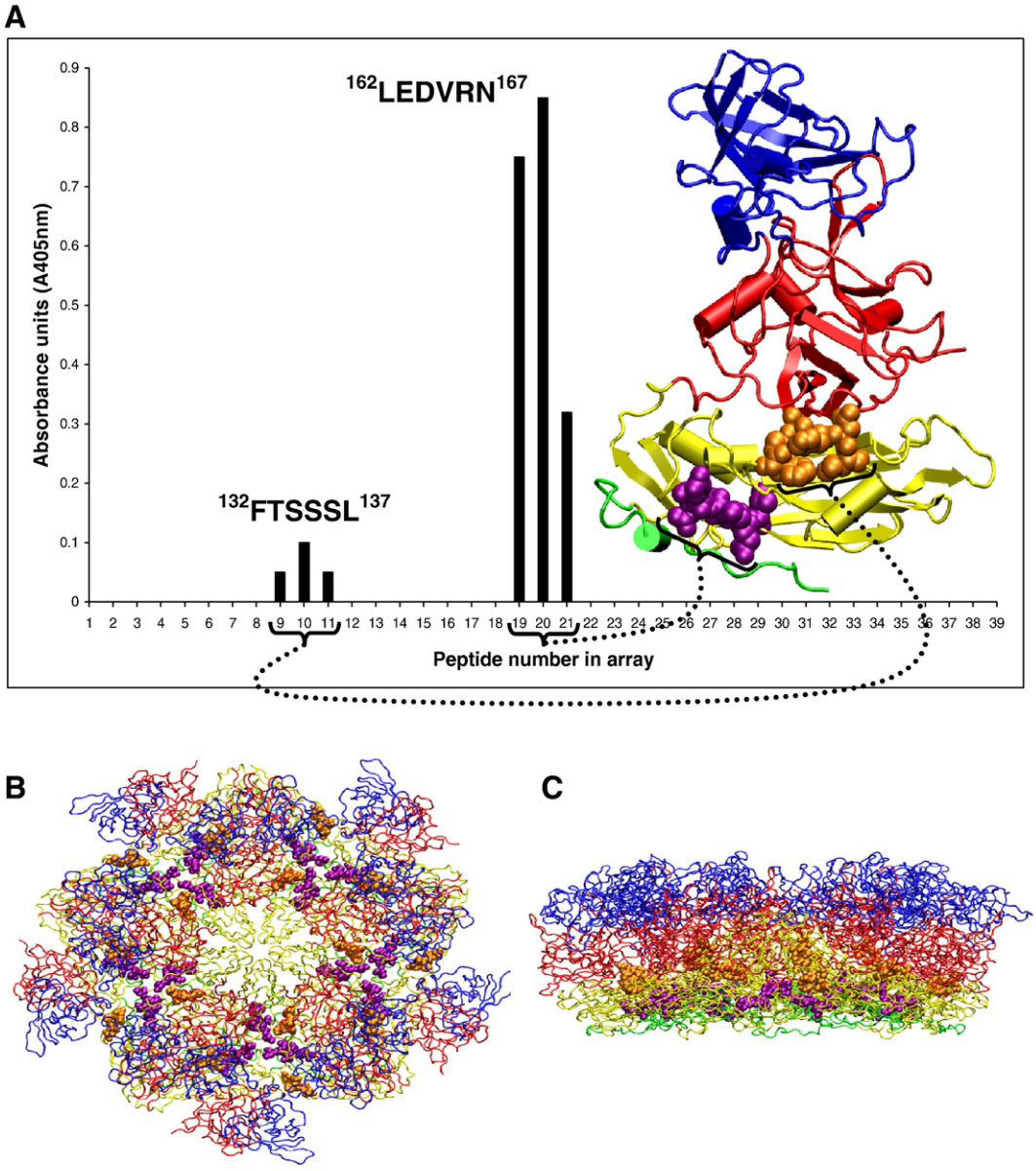

Fig. 3. High-resolution mapping and localization of the mAb CM54 epitopes. (A) Immunoreactivity by ELISA of mAb CM54 with 39 peptides generated from 123 amino acids at the NH_2 terminal of the SV capsid protein. A molecular model of the genogroup I norovirus Norwalk showing the location of the epitopes (^{132}FTSSSL^{137}; orange space fill and ^{162}LEDVRN^{167}; purple space fill) in the capsid protein monomer that reacted with the mAb CM54. Green, NH_2-terminal amino acids; yellow, S domain; red, P1 domain; blue, P2 domain. The dotted lines highlight the reactivity by ELISA of the epitopes and their location in the capsid protein. Molecular models of the five-fold axis of symmetry for the Norwalk virus viewed from the top (B) or side (C), showing the likely locations of the epitopes for CM54. The epitopes (orange and purple) are located on the inside of the viral capsid.

selection of mAbs to linear epitopes. In further studies, mAb CM54 showed broad cross-reactivity by ELISA, immunoblot and RIPA to GI capsid proteins, including Bab virus (a previously uncharacterized GI/5 norovirus) and to NA-2 (GIII/2) capsid protein. mAb CM54 did not appear to react by ELISA (data not shown) with native virus, but this probably reflects the extremely low levels of virus in some clinical specimens. Until such time as it becomes possible to cultivate human and bovine noroviruses, reactivity with native virus will depend on the chance collection of a stool sample containing high titers of virus.

The reactivities of mAb CM54 suggested it recognized a continuous (linear) epitope shared among the GI and GIII/2 noroviruses. In a separate study, strain-specific and genogroup-specific mAbs were obtained from mice immunized orally with a single recombinant norovirus VLP or two norovirus VLPs from different genogroups (Kitamoto et al., 2002) although to date the epitopes recognized by these antibodies have not been reported.

The presence of cross-reactive epitopes shared between human and bovine noroviruses has recently been reported by Oliver et al. (2006b). To add to this finding, the epitope recognized by mAb CM54 was mapped initially to low resolution by immunoblot blot analysis to truncated forms of the SV capsid protein expressed fused to GST in *E. coli*. mAb CM54 bound to a short region of the shell domain. Others have

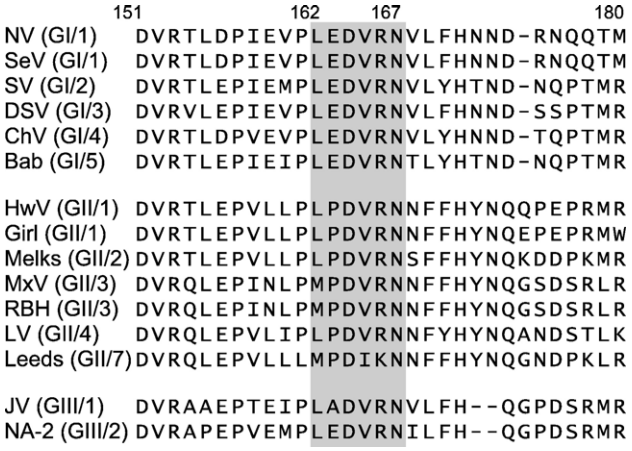

Fig. 4. Conservation of the predominant epitope LEDVRN with representatives for genogroups I, II and III of the noroviruses. Alignment of Group I, Group II and Group III norovirus partial capsid sequences between amino acids 151 and 180 of Southampton virus. The shaded box represents the epitope recognized by monoclonal antibody CM54 and is located between amino acids 162 to 167. Representative strains used for alignments are as follows: NV, Norwalk (M87661); SeV Seto (Aichi) (AB031013); SV, Southampton (L07418); DSV, Desert Shield (U04469); ChV, Chiba (AB042808); Bab, Babbacombe (AM263418); HwV, Hawaii (U07611); Girl, Girlington (AJ277606); Melks, Melksham (X81879); MxV, Mexico (U22498); RBH, RBH (AJ277617); LV, Lordsdale (X86557); Leeds (AJ277608); JV, Jena (AJ011099); NA-2, Newbury Agent 2 (AF097917).

reported a common GI epitope that was localized to a region of the P1 domain (amino acid residues 457–530) (Hale et al., 2000). mAb CM54 had reactivity to two regions of the SV capsid protein; a major epitope at amino acid residues ^{162}LEDVRN^{167} and a minor reactive epitope at amino acid residues ^{132}FTSSSL^{137}. The major epitope LEDVRN was present in the GI and GIII/2, but not GII norovirus or GIII/1 capsid proteins. It is unlikely that the FTSSSL epitope contributed to the cross-reactivity between the GIII/2 noroviruses because of the lack of amino acid conservation in this region of the capsid proteins; however, in the three-dimensional structure, these amino acid residues are in close proximity to the conserved region (LEDVRN) and may contribute to the overall affinity of mAb CM54 with its epitope.

The conservation of the LEDVRN epitope in the GI and GIII/2 noroviruses was in accordance with reactivities observed by ELISA and immunoblot to recombinant capsid proteins. Sequence alignments suggested that in the LV (GII/4), HwV (GII/1), Girl (GII/1) and Melks (GII/2) capsid proteins, an amino acid substitution from glutamate (E) to proline (P) was sufficient to prevent antibody binding by altering the overall conformation of the epitope. In addition to the glutamate to proline substitution, for MxV and RBH (GII/3) capsid proteins, the leucine (L) residue has been substituted for methionine (M). Sequence alignments with GIII norovirus capsid proteins indicated that the epitope LEDVRN was not conserved in the JV (GIII/1) capsid protein, but conserved in the NA-2 (GIII/2) capsid protein in accordance with reactivities observed by ELISA and immunoblot. JV-like (GIII/1) bovine noroviruses have been detected in the stools of calves in Michigan and Wisconsin. Alignment of partial capsid protein sequences illustrated the epitope is not conserved in the JV-like bovine noroviruses and is replaced by the sequence LADVRN (Wise et al., 2004). The single amino acid change from a glutamate (E) residue to an alanine (A) residue is likely to inhibit antibody binding. However, in NA-2-like sequences, the epitope recognized by mAb CM54 (LEDVRN) is conserved. Chakravarty et al. performed evolutionary trace analyses of noroviruses and demonstrated the presence of absolutely conserved residues (ACRs) and class-specific residues (conserved within evolutionary classes). Interestingly, D*RN residues at amino acid positions 164–167 are ACRs, while the glutamate (E) residue at amino acid position 163 is a class-specific residue in GI noroviruses (Chakravarty et al., 2005).

It is possible that mAb CM54 requires only the pentapeptide sequence EDVRN for recognition. Analysis of 103 separate protein sequences revealed, for most linear epitopes, five amino acid residues were essential to antibody binding (Geysen et al., 1988). Modeling of the amino acid sequence LEDVRN onto the predicted r-NV capsid protein structure revealed that this epitope is present on the interior surface of the virion in the S domain.

In 2005, Widdowson et al. investigated the possibility that exposure to bovine noroviruses might result in infection in humans. Human sera were tested for reactivity by ELISA, against recombinant expressed bovine norovirus capsid protein and a significant level of reactivity was found in 22% of test sera. Reactivity of IgG antibodies was detected with both r-BoV and r-NV (Widdowson et al., 2005). However, we have identified and characterized an epitope in the S domain of the capsid protein that is common to both GI and GIII/2 noroviruses. Antibodies to this epitope are cross-reactive between GI and GIII/2 by ELISA. The existence of cross-reactive epitopes between different noroviruses is therefore a factor that needs to be considered in any serological survey of human sera for antibodies to bovine norovirus. This also highlights a requirement for an assay that can distinguish antibodies to different noroviruses in polyclonal serum, in order to gain evidence of any potential transmission of these viruses between host species. This could be achieved by developing a panel of well-characterized genogroup-specific mAbs, for use in competitive ELISA to detect genogroup-specific antibodies in the test sera.

Characterization of antigenic sequences on the norovirus capsid protein will make it possible to synthesize relevant antigenic peptides. These could be used for the immunization of laboratory animals and production of mAbs and/or polyclonal antisera, for the development of improved diagnostic assays. The availability of specific antibodies with defined binding properties also provides useful tools for studying these recalcitrant viruses.

Materials and methods

Noroviruses and VLPs

Norwalk virus (NV GI/1), Southampton virus (SV GI/2) and Desert Shield virus (DSV GI/3) have been described previously (Kapikian et al., 1972; Lambden et al., 1993; Lew et al., 1994). NV was isolated from an adult during an outbreak of non-

bacterial gastroenteritis in a school in Norwalk, Ohio, USA. DSV was from military personnel during operation desert shield (1990) in Saudi Arabia. SV was obtained in 1991 from a child during a family outbreak of gastroenteritis in Southampton, UK. Babbacombe virus (Bab) was obtained from a single diarrheic stool specimen collected from an outbreak of gastroenteritis in a hotel in Babbacombe, Devon, UK, in 1996. Sequence analyses assigned Babbacombe virus to a GI/5 genotype according to the recently published classification scheme (Zheng et al., 2006). VLPs for genogroup I noroviruses were available for NV, SV and DSV (Jiang et al., 1992; Pelosi et al., 1999) (Dr. Kim Green, NIAH, personal communication), however, a new recombinant baculovirus was constructed to express the Bab capsid. The subgenomic region (ORF2-ORF3 and 3′ terminus) of Bab/96/UK norovirus was cloned and sequenced as previously described (Lambden et al., 1993). A polymerase chain reaction (PCR) fragment carrying ORF 2, 3 and 3′ UTR of Bab/96/UK norovirus was cloned into pSP73 using primer pair DORF-2 (5′-TAGTACATACTGGATCCAGC^{1}ATGATGATGGCGTCTA^{16}-3′) and *Pst*I(T)$_{20}$3UTR (5′-TAGTACATACT**CTGCAG**AGC^{2375}TTTTTTTTTTTTTTTTTTTTGGCATCAATTGTCCAAATCAA^{2334}-3′) to generate the plasmid pSP73/Bab/96/UK. The sequences in bold in primer DORF-2 indicates a *Bam*HI site and in primer *Pst*I(T)$_{20}$3UTR indicates a *Pst*I site to facilitate subcloning. The 2386-bp fragment was released from pSP73/Bab/96/UK by digestion using *Bam*HI and *Pst*I, purified and subcloned into pFastBacI to generate the plasmid Bab/96/UKpFastBacI. Transformants were screened by PCR using primers D14 (5′-^{701}TGCCCAATATACCCTTGC^{718}-3′) and D11 (5′-^{1588}CACAGGCTTTAATTGATAGAA^{1567}-3′), to confirm the presence of the insert. Recombinants carrying the correct insert were used to generate recombinant baculoviruses by co-transfection of insect cells.

Insect cell culture and the expression and purification of r-Bab VLPs was performed as previously described. VLPs were purified on cesium chloride gradients and analyzed by SDS-PAGE (Pelosi et al., 1999).

Expression of complete SV capsid protein in E. coli

A PCR fragment carrying ORF 2 of SV was amplified from pSP73/SV, a recombinant carrying the subgenomic region of the SV genome (nt 5355–6995) using primer pair SV F (5′-GACGAC**GGATCC**^{5354}ATGATGATGGCGTCTAAGGAC^{5374}-3′) and SV R (5′-GACGAC**GAATTC**^{6995}TTATATACGGCGCACTCCAAG^{6975}-3′). The sequence in bold in primer SV F indicates a BamHI site and in SV R indicates an *Eco*RI site to facilitate subsequent cloning into pGex-4T1. The purified fragment was ligated into *Bam*HI/*Eco*RI digested, dephosphorylated pGex-4T1 vector to generate the plasmid pGex-4T1/SV capsid. Transformants were screened by PCR using primers SV F and SV R and recombinants carrying the correct insert were used for expression studies. Expression of pGex-4T1/SV capsid was performed in accordance with the manufacturer's protocol (Amersham Biosciences, UK) and resulted in the formation of inclusion bodies which were purified using "Bugbuster" (Novagen) according to the manufacturer's instructions.

Expression of SV capsid protein fragments

A series of overlapping SV capsid protein fragments were generated from the recombinant plasmid pSP73/SV. PCR fragments were obtained using forward primers containing the restriction site *Bam*HI and reverse primers containing the restriction site *Sal*I or *Eco*RI as indicated in bold (Table 1). PCR products were amplified from pSP73/SV and digested with appropriate enzymes prior to ligation into appropriately digested, dephosphorylated pGex-4T1 vector to generate the plasmids pGex-SVF1, pGex-SVF2, pGex-SVF3, pGex-SVF4 and pGex-SVF5. Colonies were screened by PCR using the primers used to generate the fragments and transformants carrying the correct insert were used for expression studies. Protein expression was performed as outlined above.

Production of mAbs

Production of monoclonal antibodies against r-SV capsid protein was performed as previously described (Oliver et al., 2006b).

SDS-PAGE

SDS-PAGE was performed on protein samples using the discontinuous buffer system method of Laemmli with 12.5% acrylamide gels (acrylamide/bisacrylamide, 38.5:1 [wt/wt] (Laemmli, 1970).

Immunodetection of proteins

Proteins separated by SDS-PAGE were electroblotted to nitrocellulose sheets in transfer buffer (Tris–HCl 25 mM; glycine 192 mM; SDS 0.1% (w/v); methanol 20% (v/v)) using a Trans-Blot SD semidry blotter (Bio-Rad Laboratories Ltd., Hemel Hempstead, UK). After transfer of the proteins, the nitrocellulose membrane was washed in TTBS/5% milk (Marvel) blocking solution (TBS (NaCl 0.5 M; Tris–HCl 20 mM; pH 7.5) + Tween-20 0.05% (v/v) at 37 °C. The blocking solution was discarded and the nitrocellulose was rinsed twice with TTBS and once with TBS. mAb diluted 1/20 in 10% normal goat serum/TTBS was added to the nitrocellulose strips or sheets for 16 h. After washing in TTBS, membranes were treated with the secondary antibody (goat anti-mouse IgM alkaline phosphatase conjugate, Sigma). Alkaline phosphatase antibody conjugates were detected using a BCIP/NBT (bromo-chloro-indolyl-phosphate/nitroblue tetrazolium salt) redox chromogenic reaction catalyzed by alkaline phosphatase in a carbonate buffer ($NaHCO_3$ 0.1 mM; $MgCl_2$ 1.0 mM; pH 9.8).

Radioimmune precipitation assays (RIPA)

Full-length SV and Bab capsid genes cloned into pRSETA (Invitrogen, UK) was used as template for in vitro transcription and translation using a T7 RNA polymerase coupled reticulocyte lysate system (TNT; Promega, Southampton, UK) in

Table 1
Primers used for the production of SV capsid fragments

Clone	Fragment	Primer pair	Sequence of oligonucleotides
pGex-SV F1	SVF1	SV $F1_F$	GACTTA**GGATCC**^{5354}ATGATGATGGCGTCTAAGGAC^{5374}
		SV $F1_R$	ACGTGA**GTCGAC**^{6029}TTATATAGTAGGTGGGACAA^{6010}
pGex-SV F2	SVF2	SV $F2_F$	GACTTA**GGATCC**^{5972}GTGCTCACGGCTCCTAGT^{5989}
		SV $F2_R$	ACGTGA**GTCGAC**^{6644}TTACACAAGAGCCTCACCAAATC^{6622}
pGex-SV F3	SVF3	SV $F3_F$	GACTTA**GGATCC**^{6593}GCTAATCTGGCCCCCCCAGTA^{6613}
		SV $F3_R$	ACGTGA**GAATTC**^{6995}TTATATACGGCGCACTCCAAG^{6975}
pGex-SV F4	SVF4	SV $F1_F$	GACTTA**GGATCC**^{5354}ATGATGATGGCGTCTAAGGAC^{5374}
		SV $F4_R$	ACGTGA**GTCGAC**^{5693}TTAAAGAAGAATCCTAACTCT^{5673}
pGex-SV F5	SVF5	SV $F5_F$	GACTTA**GGATCC**^{5657}GGCTGGGTTGGAAATATG^{5674}
		SV $F1_R$	ACGTGA**GTCGAC**^{6029}TTATATAGTAGGTGGGACAA^{6010}

Nucleotides in bold indicate restriction sites used for cloning.

accordance with the manufacturer's instructions. Translation products from the TNT system (6 μl) were incubated with 2 μl of undiluted antisera or 5 μl mAb in 600 μl of RIPA buffer (10 mM Tris–HCl [pH7.5] 1 mM EDTA, 0.15 mM NaCl, 0.1% SDS, 0.5% Empigen BB [*N*-dodecyl-*N*,*N*-dimethylglycine], 0.1 mM phenylmethylsulphonyl fluoride). After incubation at 37 °C for 1 h, goat anti-mouse immunoglobulin M attached to beaded agarose (Sigma, UK) was added to adsorb immune complexes. The beads were washed three times in RIPA buffer and once in phosphate-buffered saline before solubilization of immune complexes in sample dissociating buffer and separation by SDS-PAGE. Gels were stained and prepared for autoradiography by treatment with 1 M sodium salicylate–50% methanol for 30 min at room temperature; they were then dried under vacuum and exposed to Kodak XAR-5 film at −70 °C.

Direct ELISA

Polyvinyl plates (INC Labs) were coated by incubation overnight at 4 °C with 2 μg/ml recombinant capsid protein (VLPs or GST fusion protein) in 15 mM Na_2CO_3, 35 mM $NaHCO_3$, pH 9.6/0.02% NaN_3. The wells were washed with PBS/0.05% Tween 20 (PBST) and blocked with PBS/5% (w/v) skim milk (Marvel) for 30 min at 37 °C. Hybridoma supernatants were screened at a 1:5 dilution in PBS/1% (w/v) skim milk and incubated for 1.5 h at 37 °C. After the plates were washed with PBST, 100 μl of a 1:3000 dilution of goat anti-mouse polyvalent horse radish peroxidase conjugate (Sigma, UK) diluted in PBS/1% skim milk was added and the plates were incubated for 1.5 h at 37 °C. Plates were washed with PBST before development with 100 μl substrate (0.1% sodium acetate pH 6.0, 0.1% tetra methyl benzidine, 0.01% H_2O_2) at room temperature. The reaction was stopped with the addition of 50 μl 2 M H_2SO_4 after 12 min. The absorbance at A_{450} of each well was read using an Anthos HTII plate reader (Anthos Labtec, Salzburg, Austria).

Epitope analysis

A peptide array consisting of 39 overlapping dodecamers offset by three amino acids was designed and synthesized by Mimotopes, UK. The peptides spanned amino acids 102–225 of the NH_2 terminal region of the SV capsid protein. The peptide array was synthesized on a series of solid phase 'pegs' in a 12 × 8, 96-well ELISA tray format. Epitope analysis was performed by a simple ELISA assay in accordance with the manufacturer's protocol. Briefly the pegs were submerged in a 96-well polyvinyl plate containing 200 μl precoat buffer per well (2% (w/v) BSA, 0.1% (v/v) Tween 20, 0.1% (w/v) Sodium Azide in 0.01 M PBS pH 7.2) for 1 h at room temperature. All subsequent incubations were performed by submersion in a 96-well tray. The pegs were incubated overnight at 4 °C with 200 μl per well 1/5 dilution in precoat buffer of hybridoma supernatant CM54. The pegs were washed four times for 10 min in 0.01 M PBS and submerged in 200 μl per well goat anti-mouse horseradish peroxidase conjugate, diluted in conjugate diluent (1% (v/v) Normal goat serum, 0.1% (v/v) Tween 20, 0.1% (w/v) sodium caseinate in 0.01 M PBS pH 7.2) at a working concentration of 0.25 μg/ml for 1 h at room temperature. The pegs were washed four times as previously stated. ABTS (0.05% (w/v)) was dissolved in 20 ml substrate buffer (0.1MNa_2HPO_4, 0.08 M citric acid pH 4.0, 0.01% (w/v) H_2O_2) and the pegs were incubated for 45 min with 200 μl substrate per well and the absorbance at A_{405} of each well was read using an Anthos HTII plate reader.

Molecular modeling

X-ray crystallography coordinates of the Norwalk virus capsid protein (PDB accession number **1ihm**) for the quaternary structure of the five-fold axis of symmetry were obtained from the Macromolecular Structure Database (http://www.pqs.ebi.ac.uk/). To show the location of the Southampton virus epitope sequences (^{132}FTSSSL^{137} and ^{162}LEDVRN^{167}) in the capsid protein monomer and the five-fold axis of symmetry of the Norwalk virus, image files were generated using the software program VMD 1.8.4 (Humphrey et al., 1996). Pov-ray for Windows was used to generate ray traced images for all of the structures. The domains of the Norwalk virus were amino acids 1 to 49 for the NH_2-terminal domain, 50 to 225 for the S domain, 226 to 278 and 406 to 530 for the P1 domain, and 279 to 405 for the P2 domain as previously reported (Prasad et al., 1999).

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